


414 Rec'd PCT/PTO 23 JUN 2000

FORM PCT-1376 (REV. 11-94)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 255/013 US U.S. APPLICATION NO. 09/582340
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			
INTERNATIONAL APPLICATION NO PCT/EP99/09036	INTERNATIONAL FILING DATE 23 November 1999	PRIORITY DATE CLAIMED 24/11/98	
TITLE OF INVENTION COMPOSITIONS AND METHODS USING LACTADHERIN OR VARIANTS THEREOF			
APPLICANT(S) FOR DO/EO/US Graca Raposo; Clotilde Thery; and Sebastian Amigorena			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(c)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
Items 11. to 16. below concern document(s) or information included:			
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input type="checkbox"/> Other items or information:			
CERTIFICATE OF MAILING (37 C.F.R. Section 1.10) I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office to Addressee' in an envelope addressed to the Commissioner for Patents, Box PCT, Washington, D.C. 20231.			
Express Mail No. EL496952341US Date of Deposit: June 23, 2000			


 Daniela Doss

U.S. APPLICATION NO. 097582340		INTERNATIONAL APPLICATION NO. PCT/EP99/09036		ATTORNEY'S DOCKET NUMBER 7557013 US																															
<p>17. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$760.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfy provisions of PCT Article 33(1)-(4) \$96.00</p> <p style="text-align: center;">ENTER APPROPRIATE BASIC FEE AMOUNT =</p>				<p>CALCULATIONS PTO USE ONLY</p>																															
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>CLAIMS</th> <th>NUMBER FILED</th> <th>NUMBER EXTRA</th> <th>RATE</th> <th></th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>23</td> <td>- 20 =</td> <td>3</td> <td>X \$18.00</td> </tr> <tr> <td>Independent claims</td> <td>9</td> <td>- 3 =</td> <td>6</td> <td>X \$78.00</td> </tr> <tr> <td colspan="4">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td>17</td> </tr> <tr> <td colspan="4"></td> <td>+ \$260.00</td> </tr> <tr> <td colspan="4">TOTAL OF ABOVE CALCULATIONS</td> <td>= \$1,622.00</td> </tr> </tbody> </table> <p>Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).</p> <p style="text-align: right;">SUBTOTAL =</p>				CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		Total claims	23	- 20 =	3	X \$18.00	Independent claims	9	- 3 =	6	X \$78.00	MULTIPLE DEPENDENT CLAIM(S) (if applicable)				17					+ \$260.00	TOTAL OF ABOVE CALCULATIONS				= \$1,622.00	<p>\$ 840.00</p> <p>\$</p> <p>\$ 54.00</p> <p>\$ 468.00</p> <p>260.00</p> <p>\$ 1,622.00</p> <p>\$</p> <p>\$ 1,622.00</p> <p>\$</p> <p>\$ 1,622.00</p> <p>\$</p> <p>\$ 1,622.00</p>	
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<p>Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).</p> <p style="text-align: right;">TOTAL NATIONAL FEE =</p>				<p>\$</p> <p>+</p> <p>\$ 1,622.00</p> <p>\$</p> <p>+</p> <p>\$ 1,622.00</p>																															
<p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property</p> <p style="text-align: right;">TOTAL FEES ENCLOSED =</p>				<p>\$</p> <p>+</p> <p>\$ 1,622.00</p>																															
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<p>a. <input type="checkbox"/> A check in the amount of \$_____ to cover the above fees is enclosed</p> <p>b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>12-2475</u> in the amount of \$ <u>1,622.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>12-2475</u>. A duplicate copy of this sheet is enclosed.</p> <p style="margin-top: 20px;">NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p>																																			
<p>SEND ALL CORRESPONDENCE TO: Dated June 23, 2000</p> <p>PATRICK S. EAGLEMAN</p> <p>LYON & LYON LLP</p> <p>633 West Fifth Street, Suite 4700</p> <p>Los Angeles, CA 90071-2006</p> <p>(949) 567-2300 (Telephone)</p> <p>(213) 955-0440 (Facsimile)</p>				<p style="text-align: center;"></p> <p>SIGNATURE: PATRICK S. EAGLEMAN</p> <p>NAME 44,665</p> <p>REGISTRATION NUMBER</p>																															

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(Transmittal Letter to the United States Designated Office (DO/US)—Entry Into National Stage under
35 U.S.C. 371—PTO 1390 [13-7]—page 2 of 2)

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255/013

PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICEIn re the Application of:
GRAÇA RAPOSO, et al.) **Group Art Unit:** NOT ASSIGNED) **Serial No.:** NOT ASSIGNED) **Examiner:** NOT ASSIGNED) **Filed:** HEREWITH) **For: COMPOSITIONS AND METHODS**
) **USING LACTADHERIN OR VARIANTS**
) **THEREOF****PRELIMINARY AMENDMENT**Assistant Commissioner for Patents
Washington, D.C. 20231

Sir/Madam:

Prior to the calculation of fees, please enter the following preliminary
amendments:

IN THE CLAIMS

Please cancel claim 16

Please amend claims 1-9 and 11-14 as follows:

1. [AMENDED] A method of [The use of lactadherin or a variant thereof, for the
preparation of a composition for] regulating [the] an immune response in a mammal, the
method comprising administering to the mammal a lactadherin or a variant thereof.

2. [AMENDED] The [use according to] method of claim 1, wherein [for the
inhibition of the] cross-priming of antigens is inhibited in the mammal.

3. [AMENDED] The [use according to] method of claim 2, wherein the lactadherin or a variant thereof is [of] a variant of lactadherin lacking at least one protein feature chosen from the group consisting of a functional phospholipid binding site [and/or] and a functional integrin binding site.

4. [AMENDED] The [use according to] method of claim 1, wherein [for the stimulation of the] phagocytosis of antigens by dendritic cells is stimulated in the mammal.

5. [AMENDED] The [use according to] method of claim 1, wherein [for the stimulation of the] cross-priming of antigens is stimulated in the mammal.

6. [AMENDED] [The use of lactadherin or a variant thereof, for the preparation of a] A composition for the delivery of a molecule to a dendritic cell comprising a lactadherin or a variant thereof and the molecule to be delivered.

7. [AMENDED] The [use according to] method of any one [of the preceding claims] of claims 1-5, wherein the lactadherin or variant thereof is human lactadherin or a variant thereof.

8. [AMENDED] A composition comprising [lactadherin or a variant thereof in association with a molecule] a compound of the formula L-M, wherein:
L is a lactadherin or a variant of lactadherin;
M is a selected molecule; and
wherein L is associated with M through a covalent or non-covalent linkage.

9. [AMENDED] The composition of claim 8, wherein [said molecule] L is covalently linked to M [associated to the lactadherin or variant thereof].

11. [AMENDED] The composition of claim 8, wherein M is an antigen, and the composition is [An] immunogenic [composition comprising lactadherin or a variant thereof in association with an antigen].

12. [AMENDED] A variant of lactadherin, wherein said variant lacks one or more protein features selected from the groups consisting of a functional phospholipid binding site [and/or] and a functional integrin binding site.

13. [AMENDED] A chimeric protein comprising the polypeptide sequence of lactadherin or a variant thereof fused to a polypeptide sequence.

14. [AMENDED] A nucleic acid encoding [a] the protein of [as defined in] claim 13.

REMARKS

Applicants respectfully request entry of the above amendments before calculation of fees for filing of the attached national phase application under 37 U.S.C. § 371.

Dated: June 21, 2000

Respectfully Submitted,
LYON & LYON LLP

By: 

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09/582340

COMPOSITIONS AND METHODS USING LACTADHERIN OR
VARIANTS THEREOF

The present invention relates to novel methods and composition that can be used in the biological, experimental and medical areas. More particularly, the invention relates to compositions and methods for regulating (i.e., inducing, inhibiting, stimulating, etc.) or monitoring an immune response, more specifically a CTL response, as well as for the delivery of molecules to dendritic cells, in vitro, ex vivo or in vivo.

The invention also relates to novel proteins, nucleic acids and vectors, and their use in the above discussed areas, for instance for the cross-priming of antigens, the phagocytosis of molecules by dendritic cells, etc. The present invention also provides methods of screening of molecules capable of regulating the CTL response.

Antigen recognition by the immune system can involve different mechanisms. In particular, antigen presentation to the helper T lymphocytes requires processing of said antigens by antigen presenting cells, and their association with class II molecules of the MHC complex. This recognition essentially leads to the stimulation of specific clones of B lymphocytes, which then produce antibodies specific for the antigen.

Antigen presentation to cytotoxic T lymphocytes (CTLs), on the other hand, requires the phagocytosis of the antigen by professional cells (essentially the dendritic cells), which cells contain the appropriate MHC class I molecules and are capable of stimulating antigen-specific CTLs. This phenomenon, called "cross-presentation" of antigens or "cross-priming" of CTLs occurs for instance with viral or tumor antigens, as well as with antigens contained in apoptotic bodies (resulting from viral infection, tumors, cell degeneration, or normal cell turnover, etc.) for instance.

Cross presentation of antigens is therefore a critical pathway for the elimination of antigens that are associated with pathological conditions. The capacity to induce or facilitate the cross-priming of antigens in vivo or in vitro would represent a very powerful approach to the treatment of such pathological conditions.

As indicated above, cross-priming of antigens essentially requires the phagocytosis, by dendritic cells, of particulate antigens, i.e., antigens included in bodies comprising for instance lipids, such as phosphatidyl serine. Such particulate antigens include for instance apoptotic bodies, vesicles or cell membranes. The invention now discloses the identification of a compound involved in the phagocytosis of particulate antigens by dendritic cells. More particularly, the invention discloses the identification of a compound that mediates the interaction between particulate antigens and dendritic cells, and functions as an adaptor of cross-priming.

The invention also describes novel compositions comprising a molecule associated with the above-identified adaptor or a variant thereof.

The invention can be used to regulate the immune response, in particular to reduce the immune response or, on the contrary, to facilitate (enhance) an immune response against a desired antigen. The invention also relates to novel vectors (such as proteins or liposomes) that can be used to deliver a selected molecule to a dendritic cell. The invention also relates to methods of screening of compounds that can regulate the cross-priming of antigens or the delivery of antigens to dendritic cells, as well as to methods of monitoring the immune response in a subject.

Antigen presenting cells (APCs) contain compartments of the endocytic pathway, including multivesicular bodies (MVBs), which are sites of loading of antigenic peptides on Major Histocompatibility Complex (MHC) class II molecule. These complexes are recognized by the T lymphocytes. In particular, EBV-transformed B-lymphocytes (B-EBV) have been shown to secrete such vesicles called exosomes (Raposo et al., Mol. Biol. Cell 8 (1997) 2619). B-EBV-derived exosomes contain peptide-MHC class II complexes, and are able to present them directly to CD4+ T lymphocytes (Raposo et al., 1997). More recently, vesicles secreted by another antigen presenting cells, dendritic cells (DCs), have been described (Zitvogel et al., Nature Medicine 4 (1998) 594). DCs are the only APCs able to induce primary and secondary immune responses. To do so, they have developed a remarkable array of specific attributes, which makes them a unique APC. DCs exist under two maturation states: immature DCs are efficient for antigen endocytosis and

phagocytosis, but inefficient for T cell activation, whereas mature DCs down regulate their internalization capacities, but express high levels of MHC and co-stimulation molecules, which makes them extremely efficient for T cell activation.

5 DCs-derived exosomes accumulate MHC class II and also bear MHC class I molecules. Exosomes produced by DCs exposed to tumor-derived antigenic peptides induce potent immune responses, leading to the rejection of established tumors (Zitvogel et al., 1998). Although the mechanisms of this spectacular anti-tumor effects are still unknown, the potency of exosome-mediated immune stimulation suggests that these vesicles may indeed
10 mediate activation of T cell responses physiologically.

In an attempt to understand the molecular bases of the effects of DCs-derived exosomes on the immune system, the Applicants have determined the conditions of their production by DCs, and analyzed their protein composition.

15 The results obtained show that exosomes secretion is down modulated upon DC maturation, and that exosomes concentrate a limited number of proteins. In particular, besides MHC class II molecules, one major adhesion and targeting membrane protein has been identified, which behaves as the molecular effector of the effects of exosomes on the immune system, and functions to
20 address exosomes to a cellular target, in vivo.

More particularly, the invention now demonstrates that lactadherin (also known as MFG-E8) is produced by dendritic cells, exposed at the surface of exosomes, and is involved in the phagocytosis of particulate antigens by dendritic cells. The invention also discloses variants of lactadherin, that
25 behave as competitors of lactadherin and can be used to inhibit the phagocytosis of particulate antigens by dendritic cells. The invention also provides novel methods of delivering of molecules to dendritic cells, using vectors derived from lactadherin or variants thereof.

Mature human lactadherin is a 364 amino acid protein. Furthermore,
30 lactadherin is a secreted protein and comprises a secretion signal. SEQ ID NO:1 represents the amino acid sequence of human lactadherin. Residues 1 to 23 (underlined) represent the secretion signal, and residues 24-387 represent the primary structure of the mature, secreted protein. The

murine, bovine and porcine lactadherin have also been identified, isolated and sequenced (see for instance Stubbs et al., PNAS 87 (1990) 8417 ; Hvarregaard et al., Eur. J. Biochem. 240 (1996) 628). The sequence of the murine lactadherin is also represented on SEQID NO:3. Amino acid residues 1-22 represent the secretion signal, and residues 23-463 represent the primary structure of the mature, secreted protein. Residues 111-147 are deleted in splicing variants of murine lactadherin.

Lactadherin has been originally described at the surface of milk fat globules (see Stubbs et al., *supra*). Also, this protein has been found so far essentially in mammary tissue and in cultured mammary epithelial cells. Except for their presence during lactogenesis, no function had been reported in the literature for lactadherins.

The invention now provides evidence that lactadherin is selectively produced by dendritic cells, more particularly immature dendritic cells, exposed at the surface of antigen vesicles and functions as an adaptor in antigens cross-presentation to CTLs. The invention therefore provides compositions and methods of use of lactadherin or variants thereof in the mediation of an immune response. The invention also discloses variants of lactadherin, which can be used to inhibit the cross priming, or to deliver antigens to antigen presenting cells, in particular to dendritic cells. The invention also discloses compositions comprising lactadherin or variants thereof in association with selected molecules, in particular hybrid molecules and/or liposome compositions comprising recombinant lactadherin molecules.

More particularly, it is an object of the present invention to use lactadherin or a variant thereof for the regulation of an immune response in vitro, ex vivo or in vivo. It is a particular object of this invention to use lactadherin or a variant thereof for the preparation of a composition for regulating an immune response, in particular a CTL response. It is another particular object of this invention to use lactadherin or a variant thereof for the inhibition of the cross-priming of antigens, in particular of the phagocytosis of antigens by dendritic cells.

Lactadherin comprises several functional domains. In particular lactadherin comprises two EGF-like domains, with a RGD sequence, which is

involved in integrin binding. Integrin (and more specifically integrin ($\alpha\text{v}\beta 5$) being present at the surface of dendritic cells, this region of lactadherin is involved in the interaction of this protein with dendritic cells. This interaction furthermore appears to be selective. Lactadherin also comprises a domain
5 homologous with the C-terminal region of human coagulation factors VIII and V, which represents a binding site for phospholipids, and in particular, phosphatidyl serine.

The invention now postulates and discloses that lactadherin interacts with particulate antigens, through the phospholipid binding sites, and with
10 dendritic cells, through the RGD domain, and therefore mediates the phagocytosis of said particular antigens.

It is an object of the present invention to use lactadherin, or variants thereof, more particularly human lactadherin or variants thereof, to produce cytotoxic T lymphocytes specific for a selected antigen.

15 It is another object of the present invention to use lactadherin or variants thereof, more particularly human lactadherin or variants thereof, to deliver an antigen to dendritic cells.

Within the context of the present invention, lactadherin means a protein of mammalian origin, expressed at the surface of milk fat globules, and
20 comprising a RGD site and a domain homologous to the factor VIII C-terminal region (i.e., a factor VIII-like domain). More specifically, the protein is of human, bovine, murine or porcine origin, more preferably of human origin. In a particular embodiment, lactadherin means human lactadherin having the sequence of SEQ ID NO: 1 or any functional analogs thereof. The term
25 functional analogs designates natural analogs, resulting for instance from the polymorphism or from post-translational modifications, in particular from splicing(s) or single amino acid deletion, substitution or addition, with reference to SEQ ID NO: 1, and which retain at least one functional property of human lactadherin. Functional analogs also include lactadherin of other species, in
30 particular murine lactadherin of SEQ ID NO:3.

Lactadherin used in the present invention can be either isolated from natural sources, or produced by recombinant DNA technology, as will be described below, for instance.

A "variant" of lactadherin, within the context of the present invention, means an artificial protein, i.e., a protein artificially created, and not found as such nature. Such a variant generally retains at least one biological property of lactadherin, for instance a binding property of lactadherin, and usually
5 comprises one or several of the following alterations of the primary structure:

(a) a deletion of one or several amino acid residues, more preferably several contiguous amino acid residues. In a preferred embodiment, a deletion variant comprises a deletion of several amino acid residues, which overlap at least in part with a domain of lactadherin involved in the binding of
10 phospholipids (PL), in particular phosphatidyl serine (PS), and/or integrin. The deletion can comprise up to 250 amino acids, preferably up to 200 amino acids, for instance. Furthermore, several deletions can be performed in one variant;

(b) a mutation in one or several amino acid residues, more preferably in at least one amino acid residue involved in the PS or integrin binding site of
15 lactadherin. The mutation(s) can be conservative or non-conservative. Preferably, the mutation(s) in the binding site render(s) said binding site non-functional. More preferably, a variant of the invention comprises between 0 to 1 0 point mutations, even more preferably, 0 to 5;

(c) an addition of one or several amino acid residues. The added residues are preferably located on the N- or C- terminal end of the variant, although internal residues may also be added. The added residues can have different functions. For instance, they can represent tags that can be used in a purification method (GST, myc, etc.). The added residues can also be
20 adaptors, that can be used for the association of the variant to a selected molecule such as an antigen, a nucleic acid, a receptor or a membrane lipid. Such adaptors can be for instance polylysine, other basic amino acids, a spacer, or any other DNA binding motif. The adaptor can also be a trafficking signal, such as a transmembrane domain of a protein or a lipid, for instance.
25 Furthermore, the added residues can also represent a selected molecule, fused to the factadherin, as will be discussed in more details below.

Also, as indicated above, variants according to this invention can combine one or several modifications of one or several of the above types.

A particular variant of lactadherin of this invention is a variant lacking a functional PL binding site (in particular a functional PS binding site) and/or a functional integrin binding site.

5 Variants lacking a functional PL binding site are essentially incapable of efficiently binding phospholipids such as phosphatidyl serine. Such variants however, may retain a functional integrin binding site and thus behave as competitors of natural lactadherin. Variants lacking a functional PL (i.e., phosphatidyl serine) binding site can be obtained by different methods. In a particular embodiment, said variants lack all or part of the phosphatidyl serine (PS) binding site. In another particular embodiment, such variants comprise a mutated PS binding site. Of course, variants of this invention can also comprise both deletion and mutation in the PS binding site. The phosphatidyl serine binding site of lactadherin essentially lies in the C-terminal amino acid residues of lactadherin, more particularly in the 150 C-terminal amino acid residues. A variant according to this invention is therefore any variant of lactadherin having a mutation and/or a deletion in/of the 150 C-terminal amino acid residues, and essentially incapable of binding phosphatidyl serine. A more preferred variant is a polypeptide comprising the sequence of SEQ ID NO:1, said sequence further comprising a mutation and/or a deletion in any one of amino acid residues 242 to 387.

10 In a particular embodiment of this invention, the variant comprises a deletion of at least 5, preferably at least 10, even more preferably at least 20 contiguous amino acids of the above C-terminal region. In a particular embodiment, the deletion encompasses the entire 150 C-terminal amino acid residues, more preferably the amino acid residues 242 to 387 of SEQ ID NO:1. Another specific embodiment of this invention is a lactadherin lacking the 25 C-terminal amino acids, in particular a polypeptide comprising amino acid residues 24-363 of SEQ ID NO: 1 or a polypeptide comprising amino acid residues 23-439 of SEQ ID NO:3.

30 Examples of mutations include any suppression or substitution of one or several single amino acid residues in the 150 C-terminal region of lactadherin. The substitution can be either conservative or non-conservative, preferably nonconservative.

Particular examples of mutated variants of this invention include any lactadherin having one or several point mutation(s) in the 25 C-terminal amino acids, and essentially incapable of binding PS. More particular examples are polypeptides of SEQ ID NO:2 or 4, having one or several point mutation(s) in the 25 C-terminal amino acids.

These variants of the present invention are particularly interesting since they are capable of binding integrin $\alpha v \beta 5$, i.e., dendritic cells, more preferably immature (human) dendritic cells, while they are essentially incapable of binding PS. These variants are particularly suited for the delivery of molecules to dendritic cells, in particular for the delivery of antigenic molecules to dendritic cells. These variants can also facilitate the cross-presentation of a selected antigen by dendritic cells, and thus the development of a specific CTL response against a selected antigenic molecule.

Variants of lactadherin lacking a functional integrin binding site are essentially incapable of binding efficiently dendritic cells. Such polypeptides can be any variant of lactadherin lacking all or part of the integrin binding site or which comprise a mutated integrin binding site. The integrin binding site of lactadherin essentially resides in an RGD motif located in the N-terminal part of the molecule. For instance, the integrin binding site of human lactadherin of SEQ ID NO:1 lies in residues 46-48 and the integrin binding site of murine lactadherin of SEQ ID NO:4 lies in residues 87-89. The invention therefore resides, in any lactadherin comprising a mutation in and/or a deletion in or of the RGD motif, and which is essentially incapable of binding dendritic cells. Particular examples of such variants are polypeptides of SEQ ID NO:1 which lack one, two or all of the amino acid residues RGD at position 46-48, and polypeptides of SEQ ID NO:4 which lack one, two or all of the amino acid residues RGD at position 87-89.

Such variants can be used for instance to interfere with the cross-priming of antigens, in vitro, ex vivo or in vivo, by competing with naturally occurring lactadherin for the binding to particulate antigens. Other types of inhibitors are represented by polypeptides comprising essentially the phospholipid binding site of lactadherin, for instance peptides comprising essentially the amino acid residues 364-385 of SEQ ID NO:1, or variants

thereof. Other inhibitors are represented by antibodies specific for lactadherin, in particular for lactadherin PL or integrin binding sites. Such antibodies (either polyclonals or monoclonals) can be raised by immunisation of an animal using corresponding peptides, and used to reduce an immune reaction.

5 Accordingly, an object of the present invention resides also in a variant of lactadherin which lacks a functional PL or integrin binding site.

The above variants can be prepared according to conventional techniques, known to the skilled artisan, and disclosed in more details in the Examples. For instance, they can be produced by recombinant DNA techniques, starting from a nucleic acid molecule encoding lactadherin. More particularly, a nucleic acid construct encoding lactadherin as defined above can be provided and modified according to conventional techniques to introduce mutation(s) and/or deletion(s) therein. Such techniques include digestion with restriction enzymes and/or exonucleases, ligation, site-directed mutagenesis, amplification, artificial nucleotide synthesis and the like. Such modifications can be performed in vitro, on isolated nucleic acid, generally produced or replicated in a competent host cell, such as eukaryotic or prokaryotic cells, or obtained by amplification with specific primers from a dendritic cell, for instance. In this regard, the invention also relates to a nucleic acid encoding a polypeptide as defined above. Said nucleic acid can be a DNA or a RNA molecule, or a synthetic nucleic acid. The invention also relates to any vector comprising a nucleic acid as defined above. Such vectors can be plasmids, cosmids, episomes, YACS, viral vectors, etc. Preferred vectors also comprise a promoter region that causes expression of the nucleic acid, as well as, optionally, a transcription termination signal (i.e., a polyadenylation sequence). The construction of such vectors and the choice of the promoter region can be made by the skilled artisan according to conventional techniques and routine experimentation. In particular, the vector can be a plasmid derived from pUC, pIC, pBR or pBS, or a viral vector prepared from retroviruses, adenoviruses or AAV, for instance. The invention also relates to a cell comprising a nucleic acid or a vector as defined above, and to their use, for instance in the production of variants -as described above. The recombinant cell of the present invention can be prokaryotic (such as a

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bacteria) or eukaryotic, such as a yeast cell, a mammalian cell, an insect cell, etc. Said cells can be prepared by conventional techniques (electroporation, precipitation, gene gun, lipid-mediated transfection, etc.).

5 Another object of this invention lies in a composition comprising a variant of lactadherin which lacks a functional PL or integrin binding site. Such a composition can be used for instance to modulate the cross-priming of antigens.

A particular object of this invention lies in a composition comprising lactadherin or a variant thereof in association with a selected molecule.

10 Indeed, the invention also lies in the use of lactadherin or variants to deliver molecules to dendritic cells.

In this respect, any selected molecule can be associated to lactadherin or a variant thereof as disclosed above, in order to deliver said molecule to dendritic cells in vitro, ex vivo or in vivo, and/or to induce a CTL response. The
15 selected molecule can be associated to lactadherin or a variant thereof in different manner, covalently or non covalently. Covalent association (binding) can be obtained either by chemical, enzymatic or genetic methods. In particular, chemical coupling can be made using conventional chemical adaptors and/or reactions, in particular by amide, amine, or acid bounds, with
20 or without spacers such as cabonydiimidazole, etc:

Chemical coupling can also be obtained using the streptavidin / biotin system or any other analogous method.

One particular method of coupling the selected molecule to the lactadherin involves recombinant DNA constructs (i.e., genetic coupling). In
25 this regard, a chimeric nucleic acid can be prepared comprising a region encoding lactadherin or the variant thereof and a region encoding the selected polypeptide, operably linked together, i.e., in the same reading frame, so that, upon expression in a host cell, a fusion (chimeric) protein is obtained. The selected polypeptide (or peptide) can be fused to lactadherin or a variant
30 thereof in N-terminal or C-terminal position. Preferably, the selected polypeptide or peptide (i.e., the heterologous moiety) is fused at the C-terminal end of lactadherin or a variant thereof according to this invention.

Accordingly, a particular object of the present invention resides in a chimeric protein (or polypeptide) comprising lactadherin or a variant thereof fused to a heterologous polypeptide or moiety.

Another particular object of the present invention lies in a product
5 comprising lactadherin or a variant thereof coupled to a heterologous molecule. As indicated above, coupling can be covalent or non covalent.

Also, since lactadherin mediates the phagocytosis of particulate antigens, the invention also discloses artificial liposomes (or lipid vesicles) comprising lactadherin (preferably recombinant) or a variant thereof as
10 disclosed above.

Lactadherin can be bound to the surface of the liposome, through its PL binding site. In this embodiment, the artificial liposome of this invention preferably comprises at least phosphatidyl serine as a constituent lipid. Lactadherin or the variant thereof can also be bound to the liposome
15 membrane through a receptor moiety, capable of binding a ligand moiety present in the membrane of the liposome. In particular, as indicated above, a variant of lactadherin can be prepared comprising added residues or structure, which provide for the binding of said lactadherin variant to a selected receptor or ligand. In a particular embodiment, the variant of lactadherin comprises a
20 peptide which binds a receptor present in the liposome membrane. The peptide can be biotin, which binds to streptavidin introduced in the liposome membrane.

In another particular embodiment, lactadherin or the variant is enclosed in the membrane of the liposome. The insertion (anchoring) can be achieved
25 in particular by using a variant of lactadherin comprising an anchoring region, such as for instance a transmembrane domain. Such an anchoring region can be introduced in the variant as added residues in addition or in replacement of residues of lactadherin, and in particular in replacement of the PL binding site. The insertion can also be achieved by using a variant of lactadherin
30 comprising a lipid at one of its extremities, preferably its C-terminal end, according to methods known to the skilled artisan. In this specific embodiment, a lipid (e.g. a fatty acid) is bound to the C-terminal end of

lactadherin or a variant thereof having the capacity to bind integrin, using conventional method, and the resulting product is contacted with the liposome.

Thus, the invention also relates to any lipid vesicle composition comprising a recombinant protein comprising at least the integrin binding site of lactadherin, more preferably of human lactadherin. The lipid vesicle can be prepared according to known techniques. For instance, the selected lipids are contacted together in the appropriate medium to form lipid bilayers and vesicles. Lactadherin or the variant thereof can be added to the product before, during or after formation of the artificial liposomes. Also, an artificial liposome includes an exosome produced by a cell, which comprises a recombinant lactadherin molecule.

The invention also relates to a polypeptide comprising (a) a lactadherin amino acid sequence lacking a functional PL binding site and (b) a heterologous moiety allowing anchoring of said polypeptide in a lipid membrane.

The liposome compositions of this invention advantageously further comprise at least one selected molecule, such as an antigen. The selected molecule can be a lipid, polypeptide, nucleic acid, chemical molecule, etc.

Preferably, the selected molecule is a polypeptide (such as any protein, peptide, etc.) or a nucleic acid.

In this regard, the invention also relates to a polypeptide comprising (a) a lactadherin amino acid sequence lacking a functional PL binding site and (b) a heterologous moiety allowing binding of said polypeptide to a nucleic acid. Such a polypeptide is able to form complexes with nucleic acids, such as plasmids, for instance, and to target said nucleic acids to dendritic cells in vitro, ex vivo or in vivo. This invention also relates to a nucleic acid encoding the primary structure of the above polypeptide.

Even more preferably, the molecule is an antigen, i.e., a compound against which an immune response is sought, particularly a CTL response, a cytokine (such as IL12, IL4, GM-CSF, etc., which can stimulate the activation and/or differentiation of dendritic cells, for instance) or a toxic molecule, which may be used to eliminate dendritic cells.

Examples of antigen molecules comprise for instance viral proteins or peptides, bacterial proteins or peptides, or tumor antigens, such as MART-1, MAGE, BAGE, PSA, p53, Rb, Ras, etc. The antigen can be intact (the entire proteins) or in the form of a fragment thereof.

5 A preferred embodiment of the present invention is an immunogenic composition comprising lactadherin or a variant thereof in association with a selected antigenic molecule. Vaccines or immunogenic compositions of this invention can be used in any mammal, such as human beings or animals.

10 The antigenic molecule may be any protein, polypeptide or peptide, bearing one or several epitopes, a virus, a fragment of a virus, a viral protein, polypeptide or peptide, a bacteria, etc. Furthermore, the virus or bacteria may be inactivated or attenuated (i.e., with low infectivity or pathogenicity). The virus or bacteria may represent the antigen itself, or a carrier of the antigen. In a particular embodiment, the invention thus resides in an immunogenic
15 composition comprising a virus or a bacteria, wherein said virus or bacteria expresses lactadherin or a variant thereof at its surface. Lactadherin or a variant thereof can be expressed as a result of chemical, genetic, enzymatic or non-covalent coupling.

20 In a particular embodiment, the invention resides in a recombinant virus, wherein said recombinant virus expresses lactadherin or a variant thereof as a surface molecule. The virus may be any retrovirus, adenovirus, AAV, herpes virus, etc. Lactadherin or the variant may for instant be expressed as a genetic fusion with an envelope protein. The recombinant virus may further comprise a recombinant genome encoding a protein, polypeptide or peptide, including an
25 immunogen or an immuno-stimulatory molecule.

30 In another particular embodiment, the invention resides in a recombinant cell, wherein said recombinant cell expresses lactadherin or a variant thereof as a surface molecule. More preferably, the recombinant cell is derived from a pathogenic bacteria. These recombinant bacteriae thus represent improved immunogenic compositions which can be used to produce efficient immune reactions in vivo or in vitro, in particular efficient CTL responses. The recombinant cells may be produced by expression, in said cells, of a nucleic acid encoding lactadherin or a variant thereof, optionally

bound to a signal peptide allowing anchoring of the molecule into the cell membrane. Lactadherin or the variant may also be expressed through contacting of the cells in vitro with lactadherin or the variant.

In this regard, the invention also relates to a hybrid polypeptide
5 comprising (a) a lactadherin amino acid sequence lacking a functional PL binding site fused to (b) an antigenic polypeptide or peptide. The invention also relates to any nucleic acid encoding such a hybrid polypeptide. Said polypeptides and nucleic acids can be used to prepare vaccine compositions, in particular anti-tumor vaccine compositions, which can be administered in
10 naked form, for instance by intramuscular injection.

The selected molecule can be present in the cytosol of the liposome and/or anchored into its membrane and/or bound to the surface of said liposome. The selected molecule can be combined with the liposome according to conventional methods as disclosed above, in particular by
15 incubation of the molecule with the constituent lipids, prior to or during formation of the liposome.

The invention also relates to a recombinant host cell, in particular a recombinant bacteria, expressing a lactadherin or variant thereof. More preferably, the recombinant bacteria expresses lactadherin or a variant thereof
20 having the capacity to bind integrin. The introduction of recombinant lactadherin or variant in bacteria, for instance, allows the uptake of bacterial membrane fractions by dendritic cells, and the efficient generation of immune responses against bacterial antigens, for instance.

The compositions, variants, chimeric proteins or liposomes of this
25 invention can be used in vitro, ex vivo or in vivo. In vitro, they can be used to pulse or sensitize dendritic cells to a desired molecule, for instance. In this respect, the invention also discloses a method of delivering a molecule to a dendritic cell, preferably an immature dendritic cell, in vitro, ex vivo or in vivo, comprising contacting said dendritic cell (or a population of cells comprising a
30 dendritic cell) with a compound, composition or chimeric protein as defined above. This method can be performed in any appropriate device (plate, dish, tubes, flask, etc.) with concentration of lactadherin or a variant thereof ranging from 0,01 to 1 000 µg/ml, for instance.

The variants of lactadherin can also be used to target any compound to dendritic cells, in vitro, ex vivo or in vivo. For instance, the above disclosed variants of lactadherin having a functional integrin binding site can be used to target a vector (such as a viral, a polymeric, a cationic or a polypeptide vector) to dendritic cells. Particular examples include a recombinant adenovirus. Therefore, the invention also relates to the use of lactadherin or a variant thereof having a functional integrin binding site to target a vector (such as a viral, a polymeric, a cationin or a polypeptide vector) to dendritic cells. The invention also discloses a composition comprising a vector associated with lactadherin or a variant thereof.

The compositions, variants, chimeric proteins or liposomes of this invention can also be used in vitro in a method of screening and/or identifying compounds that can regulate the cross-priming of antigens and/or the phagocytosis of antigens and/or the delivery of molecules to dendritic cells. In particular, dendritic cells can be contacted with compositions, variants, chimeric proteins or liposomes of the present invention, in the presence of a candidate product or mixture, and the capacity of said candidate product or mixture to inhibit or facilitate the uptake (or binding) of said compositions can be measured.

The invention also relates to a method of screening (essentially in vitro) of compounds having the ability to induce or inhibit the expression of lactadherin. In this respect, the invention provides a method comprising (i) contacting a test compound (or mixture) with a cell culture producing a reference level of lactadherin, (ii) measuring the quantity of lactadherin produced by said cell culture, and (iii) comparing said quantity with the reference level. The cell culture can be for instance dendritic cells, preferably an established dendritic cell line. The dosage of lactadherin can be made according to conventional techniques, such as immunoassay with specific antibodies, PCR, etc. Compounds capable of increasing the expression on lactadherin may be used to stimulate the immunity of a subject, in particular the CTL response of a subject.

The invention also relates to labeled compositions or variants as described above, and their use in the monitoring of an immune response. In

particular, the invention can be used to detect the presence of exosomes in a biological sample, and to correlate the level of exosomes to a physiological state of a subject. A detection method comprises -for instance the collection of a sample (such as a blood sample of a subject), the contacting of said sample with a composition comprising lactadherin or a variant thereof having the ability to bind integrin, and the detection of the presence (and optionally the quantity) of exosomes bound to said lactadherin or variant thereof. The diagnostic of high levels of exosomes in a biological sample can be correlated with a pathological condition where exosomes are produced, for instance by dendritic cells (such as tumor, viral infection, etc.).

In vivo, the invention can be used to modulate an immune response, deliver a molecule to dendritic cells, modulate the phagocytosis of antigens and/or for cross-priming of antigens, and also for the monitoring of an immune response. In particular, the invention also relates to the use of lactadherin, preferably human lactadherin, or a nucleic acid encoding the same, for the stimulation of an immune response in a subject. In particular, the delivery of human lactadherin or a nucleic acid encoding the same in a subject affected with a tumor would enhance the immune response of said subject against the tumor. For this specific application, intratumoral administration would be preferred.

For in vivo applications, conventional dosages can be used and adapted by the skilled artisan. Preferably, for use in vivo, the compositions are administered by injection, such as i.v., i.a., i.p., s.c., i.m., for instance. They also can be administered by any other route. Preferably, they are formulated with appropriate acceptable vehicles, such as buffers (phosphate, potassium, chloride, etc.), isotonic solutions, saline solutions, at appropriate dosage, such as for instance between 0,1 and 10000 µg of protein by single dosage unit. The compositions may also comprise stabilizing agents (such as gets, high molecular weight molecules, i.e., albumin, dextran, etc.), surfactants, etc.

Other aspects and advantages of the present invention will be disclosed in the following examples, which should be considered as illustrative and non limiting.

Legend to the Figures

Figure 1: Overall protein composition of metabolically-labeled exosomes and DI cells.

Exosomes purified from the supernatant of metabolically-labeled D1 cells were run on a 12% SDS gel (Exos), together with lysate (including both cytosolic and membrane components) obtained from the whole cells (Cells), or cytosol (cyto) or total membranes (mb) prepared from the same cells in the absence of detergent. The dried gel was autoradiographed for 24h (proteins bigger than 45 kDa) or 48 h (proteins smaller than 45kDa). A pic profile of each lane was obtained with the NIHImage software. Stars point to proteins abundant in the cells and absent in exosomes, dots to proteins enriched in exosomes.

Figure 2: Protein profile of D1 exosomes as seen by coomassie blue staining.

Thirty µg of exosomes were separated on a 8-15% gradient SDS gel, and stained with coomassie brilliant blue. The band pattern is identical to that obtained with metabolically-labeled exosomes. The major bands (1 to 11) were analyzed by trypsin digestion and MALDI-TOF (see table 1)

Figure 3: Western blot analysis of lactadherm expression in mouse DC-derived exosomes. Abundant immune reactivity was found with serum A on DC-derived exosomes, but not on whole cell lysates. Intermediate reactivity was detected on tumor cell-derived exosomes (TS/A, a mammary cell carcinoma) or in isolated membrane preparations from DCs. Annexin specific antibodies reacted with all exosome preparations and cell lysates.

Figure 4: FACscan analysis of lactadherin expression on D1 -derived exosomes. Exosome- or FCS-coated latex beads were incubated with anti-lactadherin antibodies (serum A) or the corresponding pre-immune serum. The beads were then incubated with secondary FITC labeled anti-rabbit antibodies before FACscan analysis. Abundant specific staining of exosomes coated beads was found with serum A, but not with the pre-immune serum.

Figure 5: FACscan analysis of lactadherin expression on D1 -derived
exosomes. Exosome- or FCS-coated latex beads were incubated with anti-
MHC class I, antiMHC class II, control IgG2a antibodies or anti-lactadherin
monoclonal (MC3 or MC8) or polyclonal antibodies (serum B). Antibody
binding to the beads was revealed with FITC-labeled anti-mouse or anti-rabbit
IgG antibodies (Jackson Laboratories), before FACscan analysis. Strong
staining of the exosome coated beads was found with the anti-MHC class I
and II, as well as with the different antilactadherin monoclonal and polyclonal
antibodies.

Materials and Methods

Cell lines

The spleen-derived murine DC cell line D1 has been described
previously (Winzier et al., 1997). It was cultured in Iscove's Modified
Dulbecco's Medium (IMDM, Sigma, St Quentin, France) supplemented with
10% endotoxin-free fetal calf serum (FCS, Life Technologies, Cergy, France)
and 30% R1 medium (GMCSF-transfected NIH-3T3 fibroblasts conditioned
medium). Cells were passaged twice a week in 145 mm non tissue-culture
treated Petri dishes (4-5.10⁶ cells per dish). To control their non-activated
state, the level of cell surface expression of MHC class II and costimulatory
molecules was checked regularly by fluorescence-activated cell scanning
(FACS) as described (Winzier et al., 1997).

Immun-electron microscopy

D1 cells, either immature or mature after treatment for 24 hours with
LPS were fixed with 2% paraformaldehyde in phosphate buffer 0.2M pH 7.4
(PB) for 2 hours at room temperature. Fixed cells were processed for ultrathin
sectioning and immunolabeling as described previously (Raposo et al. 1997;
Liou et al., 1996). Briefly, after washing with PB and PB-50 mM glycine, cells
were embedded in 7.5 % gelatine. Small blocks were infiltrated with 2.3M
sucrose at 4 °C for 2 hours and then frozen in liquid nitrogen. Ultrathin

cryosections prepared with a Leica ultracut-FCS (Wien, Austria) were retrieved with a mixture of Methycellulose and 2.3 M sucrose (vol./vol.) and indirectly immunogold labeled with the rat monoclonal anti-class II antibody M5114, followed by polyclonal anti-rat polyclonal antibodies (Dako, Danemark) and Protein A gold (purchased from Dr. J.W. Slot, Department of Cell Biology, Utrecht University, The Netherlands).

The sections were finally contrasted and embedded in a mixture of methylcellulose and uranyl acetate and viewed under a CM120 Twin Phillips electron microscope (Eindhoven, The Netherlands).

Exosomes purification

Exosomes were prepared either from the supernatant of a 3 days-old D1 culture, or from fresh culture medium incubated for 24h with 3 days-old D1 cells. We could not evidence any difference in the overall composition of exosomes purified from 3 days- or from 24h-supernatants. Exosomes were purified as previously described (Raposo et al., 1996). Briefly, the medium was cleared from cells and debris by 3 successive centrifugations at 300g (5 min), 1,200g (20 min) and 1 0,000g (30 min), then exosomes were recovered in the pellet after centrifugation for 1 h at 70,000g. Exosomes were washed once in a large volume of PBS, centrifuged at 70,000g for 1 h and resuspended in 50-200 ml of PBS with 0,01% sodium azide (NaN₃). The amount of exosomal proteins recovered was measured by Bradford assay (Biorad, Ivry, France).

Exosomes floatation on sucrose gradient

Ninety mg of exosomes were resuspended in 1 ml Hepes 20mM, pH 7.2 containing 2.5M sucrose and a cocktail of protease inhibitors (see next section). A continuous 2M - 0.25M sucrose gradient was loaded on top of exosomes, and this was centrifuged for 16h at 35,000 rpm in an SW41 rotor. Fractions (1 ml each) were collected from the top of the gradient, centrifuged for 1 h at 1 00,000g, and the pellet was loaded on a 1 0% SDS gel for western blot analysis.

Protein analysis by SDS-PAGE

Total proteins were prepared from 108 D1 cells lysed in 1 ml of Tris 50mM pH 7.5, NaCl 0.3M, ThionX-100 0.5%, NaN₃ 0.1%, with a cocktail of antiproteases (CLAP: chymostatine, leupeptine, aprotinin, pepstatin, 100mM each, Sigma) for 5 min at 4°C. Lysates were cleared from nuclei by centrifugation at 10,000g. To separate cytosol and total membranes, approximately 2.107 D1 cells were resuspended at 4°C in 0.3 ml of Triethanolamine 10 mM, EDTA 1 mM, acetic acid 10 mM, sucrose 250mM titrated to pH7.4 (TEA-sucrose) supplemented with CLAP, and homogenized by 60 Passages through a 25G needle. Nuclei and cell debris were cleared by centrifugation for 10 min at 1,200g. The postnuclear supernatant was centrifuged for 1 h at 100,000g: cytosol was then recovered in the supernatant, and the pellet (total membranes) was resuspended in 0,3 ml of TEA-sucrose-CLAP.

Metabolic labeling of cells and exosomes

Metabolic labeling of D1 cells was performed on 3-days-old cultures: cells were washed once in Methionine/Cysteine-free RPMI, starved for 1h at 37°C in Methionine/Cysteine-free RPMI supplemented with 30% dialysed R1 medium, and incubated overnight at 37°C in 20ml fresh Methionine/Cysteine-free RPMI-dialysed R1 supplemented with 5% endotoxin-free FCS and 10-20mCi/ml ³⁵S-Methionine/Cysteine (Promix ³⁵S, ICN, Orsay, France). Labeling medium was removed, cells were washed once in complete D1 culture medium, and further incubated for 24h in complete medium at 37°C. Exosomes were purified from the supernatant; total lysates, cytosol and total membranes were prepared from the cells. Proteins (20,000cpm of each sample) were separated on a 12% SDS-PAGE, dried and autoradiographed. The pic profile of each lane was obtained using the N1Himage software.

Protein analysis by MALDI-TOF

Thirty mg of proteins from exosomes or total lysates were run on a 6-15% SDS-PAGE, and stained with Coomassie blue (Biorad). The major bands

in the exosomes preparation were excised from the gel. Briefly, proteins were digested with trypsin for 4h at 37°C, and the peptide profile generated was analyzed by Matrix-Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-TOF,). The peptide profile of each band was compared to those of proteins in the databases using the MS-Fit program.

Western blot

Exosome preparations were resolved by SDS-PAGE and the proteins were transferred to PVDF membranes. Antibody reactivity was detected using specific secondary HRP-labeled anti-rabbit IgG antibodies (Jackson Laboratories) and ECL (Amersham).

FACscan analysis of exosomes

A new assay based on the chemical cross linking of exosomes to activated latex beads (Surfactant-free white aldehyde/sulfate latex, D : 3,9 µm, Interfacial Dynamics Corp., Portland, Or. USA) was used to detect lactadherin on DC-derived exosomes. Coating was performed by incubating 10 µl of beads with 30µg of purified exosomes for 2 h at room temperature. The exosome-coated beads were saturated in 1M glycine for 30 min at RT and incubated with the specific and FITC-secondary antibodies (anti-mouse or anti-rabbit IgG, Jackson Laboratories) in PBS 3% FCS, before analysis with a Facscalibur (Becton Dickinson).

Human DC culture

Blood cells from healthy volunteers were obtained by apheresis (Cobe Spectra cell separator). Mononuclear cells were isolated by Ficoll-Paque (Pharmacia) density gradient. Plastic adherent PBMC (peripheral blood mononuclear cells) from healthy donor were incubated for 7 days with GM-CSF and IL-4 or IL-13, in RPMI-1640 (Life Technologies) medium supplemented with 10% fetal calf serum. At the end of the culture the cells were analyzed by flow cytometry for their phenotype for CD1a, CD3, CD14, CD19, CD15, CD40, CD80, CD83, CD86, and anti HLA-DR. They exhibited phenotype characteristic of immature dendritic cells as previously described in Chapuis et al., Eur. J. Immunol, 1997, 27:431-441 (i.e. for CD1a+, CD3 -,

CD14-, CD19 -, CD15 -, CD40 +, CD80 +, CD83 -, CD86 +, and anti HLA-DR+). The exosomes were harvested from the culture supernatant as performed for exosomes from the D1 cell line.

5 Examples

1. Overall protein composition of exosomes

10 In order to understand the molecular basis of exosome's anti-tumoral effect in vivo, we undertook an extensive characterization of the protein composition of exosomes. D1 cells were metabolically labeled overnight with 35S-Methionine/Cysteine mix, washed once, and fresh non-radioactive medium was added to the cells for 24h. Metabolically labeled exosomes were purified from this supernatant. Out of 2 independent experiments, the amount
15 of radioactivity recovered in exosomes was 0.25% ($\pm 0,05\%$) of the radioactive incorporated into the cells. The amount of radioactivity recovered in exosomal proteins (80 cpm/mg) was similar to that recovered in proteins from the whole cells (100 cpm/mg), confirming that exosomal preparations are composed of cell-derived proteins, and are not contaminated by proteins from the
20 extracellular medium.

 The protein profile of exosomes from metabolically labeled cells was analyzed by SDS-PAGE and autoradiography and compared to that of whole D1 lysates, of cytosol or of total cellular membranes. As shown in figure 1, exosomes displayed a unique protein composition pattern. At least 7 proteins
25 (of approximately 180, 90, 70, 58, 43, 32 and 27 kDa see dots in figure 1, 'exos' panel) were strongly enriched in exosome preparations as compared to whole cells, cytosol or total membranes. Eight other major proteins from whole cells, cytosol or total membranes were absent or less abundant in exosomes (see stars in figure 1, 'cells' panel). Interestingly, two of the bands enriched in
30 exosomes (70 and 58 kDa) are not detected in the cytosol or the whole D 1 cells, suggesting a particularly strong mechanism of enrichment of this protein in the exosomes.

Therefore, exosomes concentrate a unique subset of cellular proteins, which most likely account for exosome's biological activities. To identify the proteins present in exosomes, thirty micrograms of exosomal proteins were loaded on a 8-15% SDS gel : the pattern of bands stained by Coomassie brilliant blue was very similar to that of metabolically labeled exosomes (compare figures 1 and 2). Therefore, all the major protein species in the exosome preparations are from cellular, and not fetal calf serum (FCS), origin. All these major bands (bands 1 to 11 in figure 2) were excised, trypsin-digested and the peptides s generated were analyzed by Matrix-Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF). The peptide profiles generated for the different bands were compared to the theoretical tryptic-peptide profiles of known proteins from the data bases. The results obtained (see Table 1 below) show that, among the identified bands, one is lactadherin (also known as Milk Fat Globule-EGF₈ Factor/VIII, MFG-E8), a peripheral membrane-associated protein.

Table 1

Band	Protein	Matching Peptide	Total Number of Peptides
4	Lactadherin	10	28
	+ hsc73	11	
5	Lactadherin	12	15

20 2. Dendritic cells but not macrophages produce Lactadherin

In view of the above evidence of the, presence of lactadherin on exosomes produced by dendritic cells, dendritic cells have been analyzed to determine if they were producing lactadherin. For that purpose, an amplification reaction has been performed on total RNA prepared from dendritic cells using oligonucleotide primers specific for the mRNA of lactadherin. The sequence of the oligonucleotides is the following:

Oligo 1 (SEQ ID NO:5): 5'-ATGCTACTCTGCGCCTCTG-3'

Oligo 2 (SEQ ID NO:6): 5'-GTCTTGTAGCTGCTGGAGG-3'

5 Total RNA was prepared from $5\text{-}7 \times 10^6$ cells by the guanidine
thiocyanate method (Chomezinski and Sacchi, *Annal. Biochem.* 162 (1987)
156). Reverse transcription was performed in a 20 μl volume: 1 μg of RNA was
mixed with dNTPs (250 μM each final), random hexanucleotides (2 ng, Gibco-
BRL), and RNasin (20 U, Promega) in a final volume of 15 μl , heated at 60°C
10 for 5 minutes, and cooled down to 42°C. Four μl of 5x AMV-Reverse
Transcriptase (RT) buffer and 1 μl of AMV-RT (25 U/ μl , Boehringer) were
added, and the reaction was performed at 42°C for 2 h. The PCR reaction
was performed in a final volume of 50 μl , using 3.5 μl of the reverse
transcription reaction, dNTPs (200 μM each final), MgCl_2 (2 mM final), Taq or
15 Tfi polymerase (Promega) and oligonucleotides specific for the mouse MFG-
E8 CDNA (500 nM each final). The mixture was heated at 94°C for 5 minutes,
submitted to 35 cycles of (94°C 30 Secondes, 53°C 30 secondes, 72°C 45
secondes), and finally to 72°C for 2 minutes. As a control, a similar PCR
reaction was set up with oligonucleotides specific for the mouse actin cDNA,
20 and submitted to 25 cycles of (94°C 30 secondes, 55°C 30 secondes and 72°C
30 secondes). Twenty μl of the PCR reactions were run on a 1 % agarose gel
containing ethidium bromide, and observed under UV illumination. The band
amplified were of 830 bp for MFG-E8, and 800 bp for actin.

25 Expression of the gene coding for MFG-E8 was analyzed by RT-PCR in
different murine cell lines: D1 (dendritic cell line), J774 (macrophage cell fine),
P815 (mastocytoma), and TS/A (mammary adenocarcinoma). D1 were used
either in their immature state, or after 24 h treatment in the presence of LPS,
that induces their maturation. Expression of MFG-E8 was detected only in
immature D1 cells and in the TS/A mammary carcinoma: neither mature D1
30 cells, nor macrophages or mastocytoma expressed MFG-E8. The good quality
of the cDNAs was shown by the amplification of an actin band from all the
samples.

3. Lactadherin expression in mouse and human DCs and DC-derived exosomes - Production of antibodies

In this example, antibodies directed against human or mouse lactadherin have been prepared. These antibodies have been used to monitor the presence and expression of lactadherin in mouse and human DC and DC-derived exosomes. These antibodies can also be used to inhibit the interaction between particulate antigens and DCs.

Three different rabbit anti-sera were raised against human and mouse lactadherin peptides. The peptides, synthesized artificially, comprise the RGD domain of lactadherin, and have the following sequence:

- CLVTLDTQRGDIFTEY (residues 79 to 94 of SEQ ID NO: 4) for the RGD domain of mouse lactadherin. This peptide was used to produce serum A.

- CEEISQEVVRGDFVPSY (residues 38 to 53 of SEQ ID NO: 1) for the RGD domain of human lactadherin. This peptide was used to produce serum B.

- a mixture of three peptides homologous in mouse and human lactadherin CEYLKTFKVAYSLLDG (residues 153 to 166 of SEQ ID NO: 1), CVTGIIITQGARDG (residues 299 to 311 of SEQ ID NO: 1) and 20 LPVS TLRL (residues 370 to 387 of SEQ ID NO: 1). These peptides were used to produce serum C.

The rabbits reacted to all peptides, except the last one.

Serum A reacted very strongly with a unique band of 50-55 kDa when exosomes from immature D1 cells or from fresh bone marrow-derived DCs were analyzed by western blot (figure 3, left panels). Reactivity was barely detectable on whole D1 cell lysates, but could be evidenced when isolated membranes were used (figure 3, right panel). Equal protein amounts, 2 or 6 µg/lane, were deposited and the quality of the deposits was evaluated using an anti-annexin II antibodies in parallel (figure 3, lower panel).

A FACscan analysis based on the chemical cross linking of exosomes to activated latex beads was used to detect lactadherin on DC-

derived exosomes. Antimouse lactadherin antibodies (serum A), but not the corresponding pre-immune serum, also reacted with exosome-coated latex beads (figure 4).

Antibodies directed to MHC class I (HC10) and H (IB5) molecules
5 reacted with human DC-derived exosome-coated beads, but not with control FCS-coated ones (figure 4). Likewise, two anti-human lactadherin monoclonal antibodies MC3 and MC8 also bind exosome- but not FCS- coated beads. Similar results were obtained using polyclonal antibodies to lactadherin (serum B), which, in contrast to the corresponding pre-immune serum, also bind to
10 exosomes (figure 5).

These results clearly show that both murine and human DC-derived exosomes bear lactadherin. The peptides and antibodies disclosed in this example can be used in other methods of this invention.

15

4. Construction of a variant of human lactadherin lacking a functional integrin binding site.

This example discloses a method for the construction of variants of
20 lactadherin lacking a functional integrin binding site. The variants obtained are essentially incapable of binding to integrin, more specifically to integrin $\alpha v \beta 5$. Said variants therefore do not efficiently bind dendritic cells, more particularly human dendritic cells, especially human immature dendritic cells. More preferably, these variants retain the ability to bind phospholipids, in particular
25 phosphatidyl serine. These molecules indeed represent competitors of naturally occurring lactadherin for the binding of particulate antigens, such as apoptotic bodies or vesicles, and interfere with the development of an immune response, especially with the cross-priming of antigens.

The variants of lactadherin lacking a functional integrin binding site are
30 constructed according to the following method.

A nucleic acid construct is provided comprising the sequence encoding human lactadherin as depicted in SEQ ID NO: 1 or at least amino acid residues 24-387 of said sequence, corresponding to the mature protein. Such

a sequence is represented on SEQ ID NO:2, for instance. Obviously, variants of said sequence can also be used as the starting material. Such a construct, generally a plasmid, is replicated in a competent host cell, in order to produce suitable quantities of nucleic acid. The nucleic acid is then treated in order to render non-functional the integrin binding site of lactadherin for which it codes. In this respect, the integrin binding site lies essentially in the N-terminal part of the protein, more particularly in the amino acid residues Arg Gly Asp at position 46-48 of SEQ ID NO:1.

In a particular example, the nucleic acid is treated by deletion of all or part of the sequence encoding amino acid residues 1 to 100 of SEQ ID NO:1, said deletion rendering the protein incapable of binding integrin $\alpha\beta 5$. Even more preferably, the nucleic acid is treated by deletion of all or part of the sequence encoding amino acid residues 1 to 100 of SEQ ID NO: 1, said deletion comprising at least all or part of the sequence encoding the amino acid residues Arg Gly Asp at position 46-48 of SEQ ID NO:1. Preferred examples of variants comprise a deletion of amino acid residues 46-48, 45-49, 40-50 or 35-55 of SEQ ID NO:1, optionally in combination with a deletion of the amino acid residues 1-23 representing the secretion signal. The deletion can be performed by various means. In particular, the deletion is performed by treatment of the nucleic acid with a restriction enzyme which cleaves said nucleic acid in the appropriate region (including at artificially created restriction sites), optionally followed by digestion of the fragments with exonucleases and/or ligation of the resulting fragments.

In another particular example, the nucleic acid is treated by mutation(s) in all or part of the sequence encoding amino acid residues 1 to 100 of SEQ ID NO:1, said mutation(s) rendering the protein incapable of binding integrin $\alpha\beta 5$. Even more preferably, the nucleic acid is treated by mutation(s) in all or part of the sequence encoding amino acid residues 1 to 100 of SEQ ID NO:1, said part comprising at least all or part of the sequence encoding the amino acid residues Arg Gly Asp at position 46-48 of SEQ ID NO:1. Preferred examples of variants comprise at least a mutation of one amino acid selected from the amino acid residues Arg Gly Asp at position 46-48 of SEQ ID NO:1. The mutation can be carried out by various means, including site-directed

mutagenesis, amplification with mutated primers, nucleic acid synthesis and cloning, etc. The mutation can lead to the suppression of one single amino acid residue or to its replacement by another conservative or non-conservative amino acid residue.

5 Once they are prepared, the structure of the nucleic acids is verified by digestion and/or sequencing. The resulting nucleic acids are then expressed in a host cell, for instance a bacteria, a yeast cell or a mammalian cell, and the variants thereby produced are recovered.

Particular variants of the present invention are:

- 10 - a protein comprising amino acid residues 49-387 of SEQ ID NO:1;
 - a protein of SEQ ID NO:1 with a deletion of amino acid residues 46-48;
 - a protein of SEQ ID NO:1 with a deletion of amino acid residues 1-23 and 46-48;
 - a protein comprising amino acids 23-387 of SEQ ID NO:1 with a
 15 mutation in amino acid residue Arg at position 46;
 - a protein comprising amino acids 23-387 of SEQ ID NO:1 with a mutation in amino acid residue Gly at position 47;
 - a protein comprising amino acids 23-387 of SEQ ID NO:1 with a mutation in amino acid residue Asp at position 48;

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5. Construction of a variant of human lactadherin lacking a functional Phosphatidyl serine binding site

25 This example discloses a method for the construction of variants of lactadherin lacking a functional Phosphatidyl Serine (PS) binding site. The variants obtained are essentially incapable of binding to PS and therefore do not efficiently bind particulate antigens, such as apoptotic bodies or vesicles. On the other hand, said variants advantageously retain the ability of lactadherin to bind dendritic cells, more particularly human dendritic cells, especially human immature dendritic cells. Accordingly, these molecules represent competitors of naturally-occurring lactadherin for the binding of dendritic cells thereby interfering with the development of an immune
 30 response, especially with the cross-priming of antigens. These variants can

also be used to deliver molecules to dendritic cells, in particular to facilitate (i.e., enhance) the uptake (e.g., the phagocytosis) of molecules by dendritic cells, in vitro, ex vivo or in vivo.

5 The variants of lactadherin lacking a functional PS binding site are constructed according to the following method.

A nucleic acid construct is provided comprising the sequence encoding human lactadherin as depicted in SEQ ID NO: 1, as disclosed in example 4. The nucleic acid is then treated in order to render non-functional the PS binding site of lactadherin for which it codes. In this respect, the PS binding
10 site lies essentially in the C-terminal part of the protein, more particularly in the amino acid residues 242 to 387 of SEQ ID NO:1, even more preferably in the amino acid residues 350-387 of SEQ ID NO:1.

In a particular example, the nucleic acid is treated by deletion of all or part of the sequence encoding amino acid residues 242 to 387 of SEQ ID
15 NO:1, more preferably comprising at least the amino acid residues 360-387 of SEQ ID NO:1, said deletion rendering the protein incapable of binding PS. The deletion can be performed by various means. in particular, the deletion is performed by treatment of the nucleic acid with a restriction enzyme which cleaves said nucleic acid in the appropriate region (including at artificially
20 created restriction sites), optionally followed by digestion of the fragments with exonucleases and/or ligation of the resulting fragments.

In another particular example, the nucleic acid is treated by mutation(s) in all or part of the sequence encoding amino acid residues 242 to 387 of SEQ ID NO:1, more preferably at least in the sequence encoding the amino acid.
25 residues 360-387 of SEQ ID NO:1, said mutation(s) rendering the protein incapable of binding PS. The mutation can be carried out by various means, including site-directed mutagenesis, amplification with mutated primers, nucleic acid synthesis and cloning, etc.

Once they are prepared, the structure of the nucleic acids is verified. by
30 digestion and/or sequencing. The resulting nucleic acids are then expressed in a host cell, for instance a bacteria, a yeast cell, an insect cell or a mammalian cell, and the variants thereby produced are recovered.

Particular variants of the present invention are:

- a protein comprising amino acid residues 1 to 242 of SEQ ID NO:1;
- a protein comprising amino acid residues 24 to 242 of SEQ ID NO: 1;
- a protein of SEQ ID NO:1 with a deletion of amino acid residues 360-387;

- 5 - a protein of SEQ ID NO:1 with a mutation in one or several amino acid residues of the sequence 360-387.

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CLAIMS

1. The use of lactadherin or a variant thereof, for the preparation of a composition for regulating the immune response.
- 5 2. The use according to claim 1, for the inhibition of the cross-priming of antigens.
- 10 3. The use according to claim 2, of a variant of lactadherin lacking a functional phospholipid binding site and/or a functional integrin binding site.
- 15 4. The use according to claim 1, for the stimulation of the phagocytosis of antigens by dendritic cells.
5. The use according to claim 1, for the stimulation of the cross-priming of antigens.
- 20 6. The use of lactadherin or a variant thereof, for the preparation of a composition for the delivery of a molecule to a dendritic cell.
7. The use according to any one of the preceding claims, wherein the lactadherin or variant thereof is human lactadherin or a variant thereof.
- 25 8. A composition comprising lactadherin or a variant thereof in association with a molecule.
9. The composition of claim 8, wherein said molecule is covalently associated to the lactadherin or variant thereof.
- 30 10. The composition of claim 8, comprising a liposome.
11. An immunogenic composition comprising lactadherin or a variant thereof in association with an antigen.

12. A variant of lactadherin, wherein said variant lacks a functional phospholipid binding site and/or a functional integrin binding site.

5 13. A chimeric protein comprising lactadherin or a variant thereof fused to a polypeptide.

14. A nucleic acid encoding a protein as defined in claim 13.

10 15. A liposome comprising a recombinant lactadherin or variant thereof.

16. A composition comprising a variant of lactadherin according to claim 12 or a chimeric protein according to claim 13 or a liposome according to claim 15.

15 17. The composition of claim 11, wherein the antigen is a protein, polypeptide, peptide, virus or bacteria.

20 18. A recombinant virus expressing lactadherin as a surface molecule.

19. A recombinant cell expressing lactadherin as a surface molecule.

ABSTRACT

5 COMPOSITIONS AND METHODS USING LACTADHERIN OR
 VARIANTS THEREOF

- 10 The present invention relates to novel methods and composition that
 can be used in the biological, experimental and medical areas. More
 particularly, the invention relates to compositions and methods using
 lactadherin or variants thereof, or a corresponding nucleic acid, for regulating
 (i.e., inducing, inhibiting, stimulating, etc.) or monitoring an immune response,
 more specifically a CTL response, as well as for the delivery of molecules to
15 dendritic cells, in vitro, ex vivo or in vivo.

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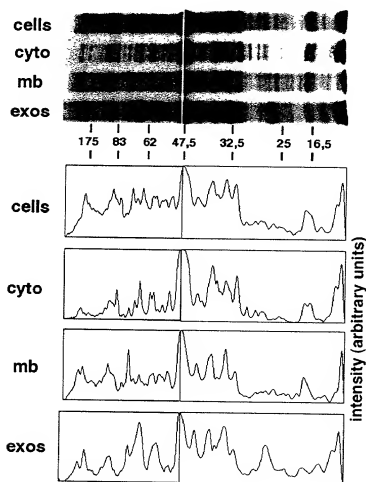


FIGURE 1

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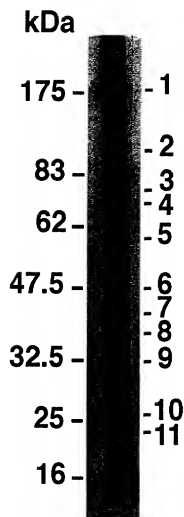


FIGURE 2

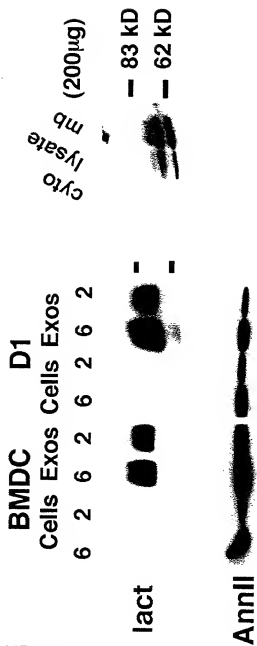


FIGURE 3

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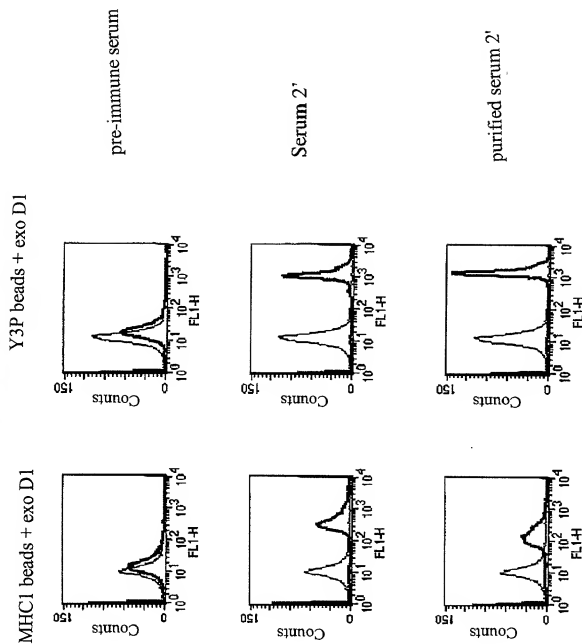


FIGURE 4



ORIGINAL

80019 wo/us
97126.01 - AMICORNTLyon & Lyon LLP
Docket Information
255/013

#3

DECLARATION
Utility Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **COMPOSITIONS AND METHODS USING LACTADHERIN OR VARIANTS THEREOF** the specification of which

(Check One)

☐
☒

is attached hereto OR
was filed on June 23, 2000 as United States Application Serial No. 09/582,340 or PCT International Application No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Date of Filing	Priority Claimed	
			Yes	No
PCT/EP99/09036	EP	November 23, 1999	✓	

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date	Status-Patented, Pending or Abandoned

**POWER OF ATTORNEY
By Assignee**

INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE, INSTITUT CURIE
and CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE, assignee(s) of the application
for United States Letters Patent for an improvement in

COMPOSITIONS AND METHODS USING LACTADHERIN OR VARIANTS THEREOF
by Graca Raposo, Clotilde Thery and Sebastian Amigorena,

the specification of which:

- ☐ is filed herewith, OR
☐ was filed on _____, having U.S. Patent Application Serial No. _____,

does hereby appoint as my attorneys and/or agents, with full power of substitution and revocation,
to prosecute this application and transact all business in the United States Patent and Trademark
Office, and in countries other than the United States, and to do all things necessary or appropriate
therefor before any competent International Authorities in connection with any international patent
application(s) corresponding to the above-identified application, all of the registered practitioners
identified by Customer Number 22249:



22249

PATENT TRADEMARK OFFICE


LYON & LYON LLP
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Please send all correspondence to the attention of Kurt T. Mulville, at the above Customer
Number, and direct all telephone calls to **(949) 567-2300**.

I, the undersigned, declare that I have reviewed copies of the documentary evidence establishing
chain of title to the patent application identified above from the inventor(s) to the assignee(s),
which:

- ☒ is filed for recordation herewith; or
☐ was recorded at Reel _____, Frame _____; or
☐ has been sent for recordation under separate cover, copy attached herewith.

To the best of the undersigned's knowledge and belief, title is in the assignee(s) identified above.
Furthermore, the undersigned is empowered to sign this document on behalf of the assignee(s).

Full Name of Assignee: Institut National de la Sante Et de la Recherche Medicale	
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If Other Than Assignee: Christine BERLIN <i>Pour le Directeur Général</i>	Head of Department, on
Title of Declarant: <i>Chef de Service</i> <i>et par délégation</i>	behalf of the General Manager
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Address of Declarant: 3 rue Michel-Ange, 75794 Paris Cedex 16, France	

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

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2-00

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3-00

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